

Activation of Innate Immunity Is Required for Efficient Nuclear Reprogramming

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<http://dx.doi.org/10.1016/j.cell.2012.09.034>

SUMMARY

Retroviral overexpression of reprogramming factors (Oct4, Sox2, Klf4, c-Myc) generates induced pluripotent stem cells (iPSCs). However, the integration of foreign DNA could induce genomic dysregulation. Cell-permeant proteins (CPPs) could overcome this limitation. To date, this approach has proved exceedingly inefficient. We discovered a striking difference in the pattern of gene expression induced by viral versus CPP-based delivery of the reprogramming factors, suggesting that a signaling pathway required for efficient nuclear reprogramming was activated by the retroviral, but not CPP approach. In gain- and loss-of-function studies, we find that the toll-like receptor 3 (TLR3) pathway enables efficient induction of pluripotency by viral or mRNA approaches. Stimulation of TLR3 causes rapid and global changes in the expression of epigenetic modifiers to enhance chromatin remodeling and nuclear reprogramming. Activation of inflammatory pathways are required for efficient nuclear reprogramming in the induction of pluripotency.

INTRODUCTION

Seminal studies by Yamanaka and colleagues revealed that ectopic expression of four transcriptional factors (Oct4, Sox2, Klf4 and c-Myc; OSKM) induces pluripotency in somatic cells (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). However, much remains to be understood about the underlying mechanisms of reprogramming of somatic cells to iPSCs. Furthermore, retroviral overexpression of the reprogramming factors raises concerns that the integration of foreign DNA into the host genome could silence or induce dysregulation of indispensable genes (Maherali et al., 2007; Okita et al., 2007; Takahashi

et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007). A protein-based approach for iPSC generation avoids all concerns for integration of foreign DNA and theoretically provides for greater control over the concentration, timing, and sequence of transcription factor stimulation (Yang et al., 2009; Zhou et al., 2009). Successful reprogramming to pluripotency has been achieved by using purified recombinant proteins or cell extracts containing these proteins, but is exceedingly inefficient (Cho et al., 2010; Kim et al., 2009; Zhou et al., 2009) (~0.001%) by comparison to reprogramming with retroviral vectors (0.1%–1%) (Stadtfeld and Hochedlinger, 2010). In seeking to develop effective reprogramming protocols with cell-permeant proteins (CPPs), we fortuitously observed an intriguing difference in the pattern of gene expression induced by viral, as opposed to CPP-based, methods. The accelerated and sustained induction of gene expression that is observed with retroviral methodology is recapitulated by combining CPPs with retroviral particles encoding nonrelevant genes or more practically by combining CPPs with agonists of toll-like receptors (TLRs). We further observed that the induction of toll-like receptor 3 (TLR3)-mediated signaling promotes epigenetic remodeling that is generally required for efficient reprogramming. Recognition of the role of innate immunity in nuclear reprogramming, and its directed manipulation, provides key insights that may facilitate our understanding of both innate immunity and reprogramming and increase our understanding of the genetic and epigenetic pathways that function in induced pluripotency.

RESULTS

Different Patterns of Gene Expression Induced by Reprogramming Factors Delivered as Viral Constructs versus Cell-Permeant Peptides

The CPPs are fusion peptides, each with a reprogramming factor, a linker, and a cell transduction domain (Yang et al., 2009). These CPPs exhibit cognate DNA-binding activity, rapidly translocate across the plasma and nuclear membranes, uniformly

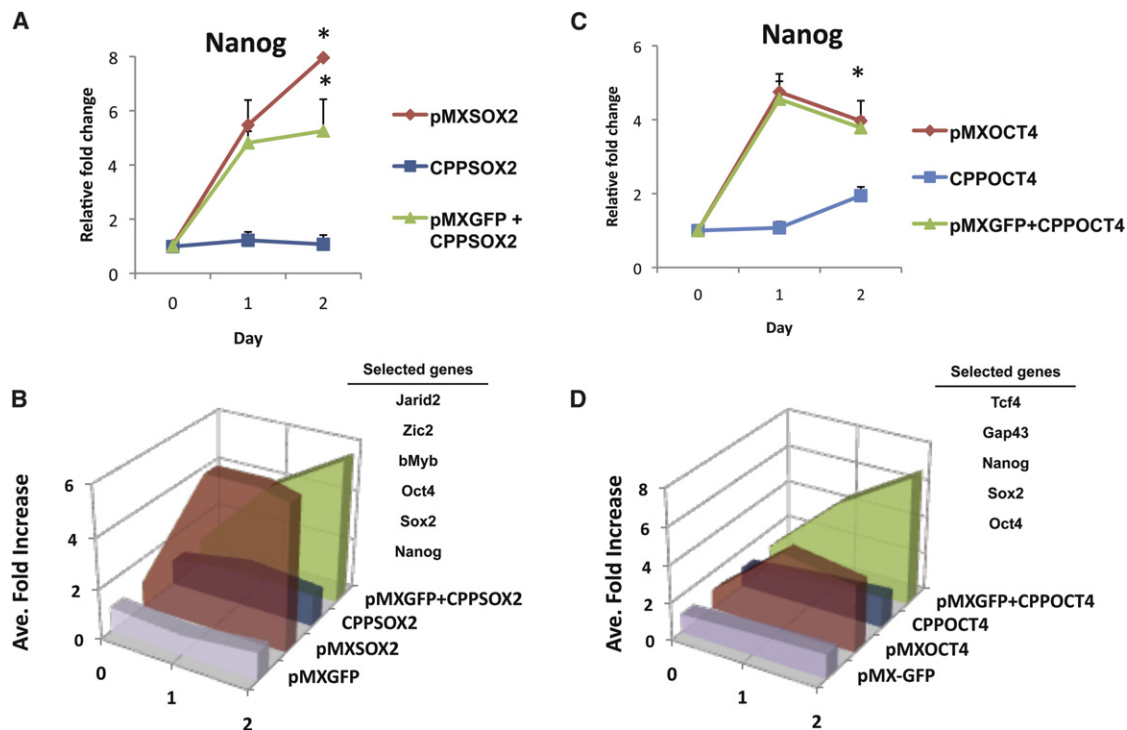


Figure 1. Irrelevant Retroviral Vector Accelerates CPP-Induced Gene Expression

Changes in expression of pluripotency genes were assessed after exposure of human fibroblasts to the retroviral vectors encoding Sox2 or Oct4 or the cell permeant peptides of Sox2 or Oct4. The addition of an irrelevant retroviral vector encoding GFP (pMX-GFP) enhanced the response to the cell permeant peptides. (A) Relative fold change in gene expression levels of *Nanog* after exposure to pMX-Sox2 (red line), CPP-Sox2 (blue line), or pMX-GFP + CPP-Sox2 (green line). Relative fold change in gene expression was determined following treatment with 200 nM CPP-Sox2 daily or after a single pMX-Sox2 infection on day 0. All data represented as mean \pm S.D., $n = 3$, * $p < 0.005$.

(B) Summary figure showing the average fold-change in the selected genes (i.e., *Jarid2*, *Zic2*, *bMyb*, *Oct4*, *Sox2*, and *Nanog*) over time for each condition. When Sox2 is given in the form of a CPP, activation of the downstream target genes is delayed. However, in the presence of an irrelevant retroviral vector, target gene expression increases rapidly and mimics that of pMX-Sox2.

(C) Relative fold change in gene expression levels of *Nanog* following pMX-Oct4, CPP-Oct4, or pMX-GFP + CPP-Oct4 treatments.

(D) Summary figure showing the average fold-change in the selected genes (i.e., *Tcf4*, *Gap43*, *Nanog*, *Sox2*, and *Oct4*) over time for each condition. All data represented as mean \pm S.D., $n = 3$, * $p < 0.005$ (see also Figures S1 and S2).

transduce nearly all cells in the treated wells, and exert transcriptional control on known downstream target genes. Nevertheless, after multiple attempts with a variety of experimental protocols, we initially failed to generate iPSC lines from human fibroblasts by using CPPs generated by our group or by commercial vendors.

In an effort to understand and overcome this hurdle, we examined the temporal sequence of target gene expression in response to a retroviral construct (pMX-Sox2) versus the corresponding CPP (CPP-Sox2). We focused on validated downstream targets such as *Jarid2*, *Zic2*, and *bMyb* for Sox2-activated genes, as well as, downstream genes known to function in nuclear reprogramming, i.e., *Nanog*, *Sox2*, and *Oct4* (Boyer et al., 2006; Li et al., 2010; Sharov et al., 2008; Tarasov et al., 2008). Human fibroblasts were synchronized by serum starvation and then subjected to either a single infection with pMX-Sox2 or daily treatments of CPP-Sox2, and gene expression was assayed up to 20 days. We used a daily dose of CPP-Sox2 (200 nM) that we had shown could rescue human iPSCs treated with shRNA against Sox2 (data not shown).

An intriguing difference in the pattern of gene expression was observed. As early as day 1 of transfection with pMX-Sox2, human BJ fibroblasts manifested increased expression of the pluripotency (e.g., *Nanog*) and target genes (Figures 1A and 1B and Figure S1 available online). By contrast, despite its rapid entry into the cytoplasm and nucleus of treated cells (within 2 hr; data not shown), CPP-Sox2 did not show a similar increase in target gene expression in the first 48 hr (Figure 1A). Because the temporal pattern of expression of the selected genes (*Jarid2*, *Zic2*, *bMyb*, *Oct4*, *Sox2*, and *Nanog*) was remarkably similar for each treatment condition, their change in fold-expression over time is shown as an average in Figure 1B. The observed difference in target gene expression occurred despite high transfection efficiency with the CPP (over 80%; data not shown).

To exclude the possibility that the delay in target gene expression was a function of the design of a single CPP, we repeated these experiments comparing a CPP versus viral vector for Oct4 (pMX-Oct4), assessing the effect on downstream targets (*Tcf4* and *GAP43*) and pluripotency genes. We observed a similar

pattern of delayed gene expression in cells treated with CPP-OCT4 compared to those transfected with pMX-Oct4 (Figures 1C and 1D). Again, because the temporal pattern of expression of the selected genes was remarkably similar for each treatment condition, their change in fold-expression over time is shown as a group average in Figure 1D. Thus, the pattern of delayed expression of downstream target genes appeared to be an attribute of CPP reprogramming factors. To determine whether this difference in gene expression was attributable to the temporal differences in the expression levels of the reprogramming factors between the two delivery methods, we quantified the nuclear expression of Oct4 after CPP or retroviral delivery of Oct4 (data not shown). Both of the delivery methods showed equal expression of Oct4 at 24 hr posttreatment.

To further determine the role of CPPs on induction of pluripotency, we checked the gene expression of pluripotency factors at later time points (days 4–20). Retroviral Oct4 induced an exponential increase in pluripotency gene expression at later time points (Figures S2A–S2C). In contrast, after an initial delay, CPP-OCT4 induced only a modest increase in pluripotency gene expression (“low and slow”).

Viral Particles Accelerate and Augment CPP-Induced Expression of Target Genes

We hypothesized that an intrinsic feature of viral particles, independent of the genes encoded, might influence the reprogramming process. To test this hypothesis, we assessed the effect of the CPPs alone or in the presence of a retroviral particle encoding a gene not involved in reprogramming. The pMX-GFP control vector did not affect target gene expression (Figure S1C). However, when the pMX-GFP vector was combined with CPP-SOX2, the expression of the Sox2 target genes was enhanced substantially. In fact, the combination reproduced the pattern of gene expression induced by the retrovirus expression vector pMX-Sox2 (Figures 1A and 1B and Figures S1A and S1B). We repeated these studies with CPP-OCT4 (Figures 1C and 1D and Figures S1D and S1E). When the pMX-GFP vector was combined with CPP-OCT4, again the temporal expression of the downstream genes was accelerated, mimicking the effect observed with the viral vector pMX-Oct4 (Figures 1C and 1D). These studies suggested that some intrinsic feature of the viral particle contributed to nuclear reprogramming.

Mechanism by which Viral Particles Enable CPP-Induced Expression of Target Genes

To gain more insight into the mechanisms by which a retroviral particle could accelerate nuclear reprogramming, we substituted a nonintegrating mutant of pMX-GFP by introducing a frameshift mutation near the *pol* coding region of the retrovirus (Hagino-Yamagishi et al., 1987). Notably, the pMX-GFP nonintegrating mutant was fully capable of accelerating the target gene expression induced by CPP-OCT4 (Figures S1F–S1J). Accordingly, integration of foreign DNA into the host genome is not required for the difference in gene expression between the CPPs and their corresponding viral vectors.

Viral infection activates innate immunity by virtue of interaction with toll-like receptors (TLRs) (McWhirter et al., 2004). The TLRs

recognize pathogen-associated molecular patterns (PAMPs) associated with viral protein, lipopolysaccharides, DNA or RNA (Akira and Takeda, 2004; Beutler, 2004; Kato et al., 2005). We hypothesized that activation of innate immunity through TLRs may be involved in the difference in gene expression. Indeed, we observed that our retroviral vectors (but not the CPPs) activated inflammatory (innate immune response) genes, including the toll-like receptor 3 (*TLR3*), *NF- κ B*, *IFN- β* , *Stat1*, and *Stat2* (data not shown).

TLR3 Signaling Enables Efficient Induction of Pluripotency Genes by Retroviral Oct4

The TLR-signaling pathway consists of two distinct pathways: a myeloid differentiation primary response gene (*MyD*) 88-dependent pathway and a *MyD88*-independent pathway (Kawai and Akira, 2010). The *MyD88*-dependent pathway is common to all TLRs, except *TLR3* (Adachi et al., 1998; Kaisho and Akira, 2006). To distinguish which TLR signaling pathway might be involved in nuclear reprogramming with viral vectors, we used inhibitory peptides or shRNA knockdown directed against the *MyD88*-dependent and -independent pathways.

The *TLR3* pathway is activated by viral dsRNA and is independent of *MyD88*. The adaptor for *TLR3* is TRIF (for TIR-domain-containing adaptor-inducing interferon- β) (Yamamoto et al., 2003). To explore the role of this pathway in the action of retroviral reprogramming constructs, we knocked down *TLR3* or TRIF. In addition, we employed a cell permeable peptide inhibitor of TRIF. As expected, peptide inhibition of the TRIF adaptor molecule or knockdown by shRNA of TRIF or *TLR3* reduced the activation by pMX-Oct4 of immune response genes (Figures S2D–S2F). Notably, these knockdowns of *TLR3* signaling also decreased the target and pluripotency gene expression induced by pMX-Oct4. The peptide inhibitor of TRIF (TRIF-Inh) attenuated the effect of pMX-Oct4 to induce Oct4 expression (Figure 2A), as well as expression of the other target genes (Figure 2D). Similarly, shRNA knockdown of TRIF (Figures 2B and 2E), as well as shRNA knockdown of *TLR3* (Figures 2C and 2F), also attenuated the effect of pMX-Oct4 to induce expression of the target genes.

By contrast, inhibition of *MyD88* by an inhibitory peptide (Figure S2G) or by a stable shRNA knockdown (Figure S2H) had no effect on the target and pluripotency gene expression induced by pMX-Oct4. Together, these studies indicate that *TLR3*, but not the other TLR pathways, are required for full induction of target gene expression by the retrovirus expression vector.

TLR3 Signaling Enables Efficient Generation of Human iPSCs by Retroviral Vectors

To determine whether *TLR3* signaling was necessary for efficient generation of human iPSCs, we assessed retroviral reprogramming in BJ fibroblasts previously treated with scrambled shRNA or shRNA to knockdown (KD) the expression of *TLR3*, *TRIF*, or *MyD88* (Figure 3A). Six days following transduction, the cells were seeded on mitomycin C-treated mouse embryonic fibroblasts (MEFs), and the following day the medium was replaced with iPSC medium. Around day 25 we observed small colonies in the Scramble- and *MyD88*-KD cells; by contrast, no colonies

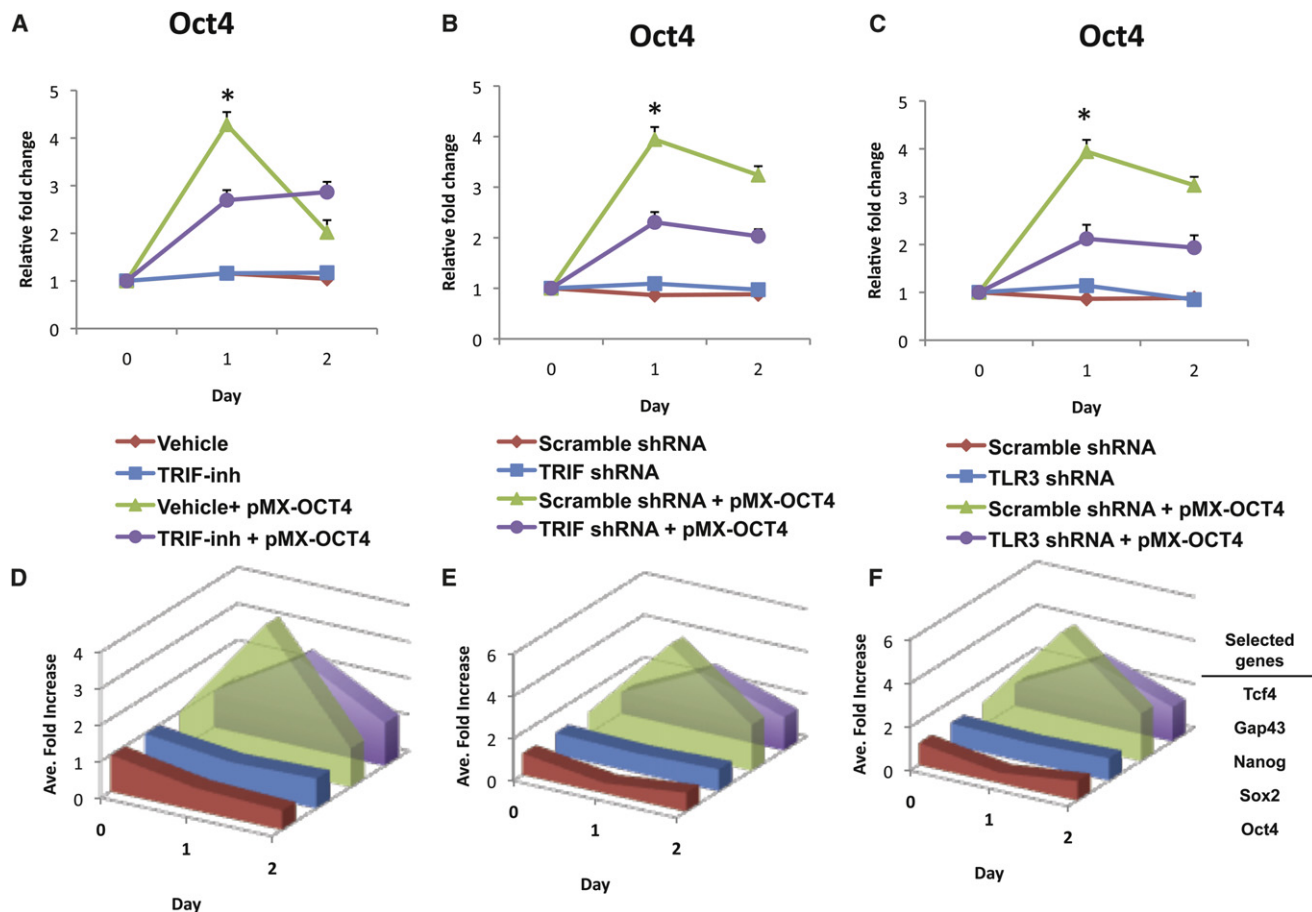


Figure 2. Knockdown of TLR3 Pathway Inhibits Action of Retroviral Vector Encoding Oct4

(A) Gene expression of *Oct4* following retroviral-Oct4 (pMX-Oct4) infection is reduced in fibroblasts treated with the TRIF-inhibitory peptide (TRIF-inh). (B) Gene expression of *Oct4* following retroviral-Oct4 (pMX-Oct4) infection is reduced in TRIF shRNA-knockdown fibroblasts. Lower: summary diagram of the average fold-changes over time in the selected pluripotency genes (*Oct4*, *Sox2*, and *Nanog*) in the four conditions. (C) Gene expression of *Oct4* following retroviral-Oct4 (pMX-Oct4) infection is reduced in TLR3 shRNA-knockdown fibroblasts. (D–F) Summary figures showing the average fold-changes in the selected genes (i.e., *Tcf4*, *Gap43*, *Nanog*, *Sox2* and *Oct4*) over time for each condition. All data represented as mean \pm S.D., $n = 3$, * $p < 0.005$ (see also Figure S2).

were observed in the TRIF- or TLR3-KD cells. By day 30, distinct colonies with typical iPSC colony morphology were noted in dishes containing the scramble- and MyD88-KD cells (Figure 3B). At this time, the TRIF- and TLR3-KD cells manifested only small granulated colonies. It took another 9 days for the TRIF- and TLR3-KD cells to yield morphologically distinct iPSC colonies (data not shown). We manually counted each distinct colony with typical morphological features, as well as those smaller granulated colonies, appearing from days 30–39 (in two independent experiments by an observer blinded to the treatment group). As seen in Figure 3C, at early time points the TRIF- and TLR3-KD cells generated fewer colonies by comparison to scramble- and MyD88-KD cells. Furthermore, we compared the gene expression values between these colonies at day 30. The expression of *Oct4* (Figure 3D), *Sox2*, and *Nanog* data not shown, were upregulated more than 10-fold when compared to the TRIF- and TLR3-KD iPSCs. These findings provide evidence that TLR3 activation is necessary

for efficient induction of pluripotency genes and generation of human iPSC colonies by using the approach first described by Yamanaka.

TLR3 Signaling Enables Efficient Generation of Human iPSCs by mmRNA Encoding Reprogramming Factors

To determine whether TLR signaling was required for other methods of reprogramming, we assessed the effects of TLR knockdowns on mmRNA-based reprogramming. The scramble or KD human BJ fibroblasts were treated with modified messenger RNA (mmRNA) encoding OSKM (Figure 4A). We observed that when the TLR3 pathway is knocked down (by using shRNA against TLR3 or TRIF), expression of the pluripotency genes in single colonies is reduced (Figure 4D). Furthermore, we find that TRIF- and TLR3 KD cells generated fewer colonies by comparison to scramble- and MyD88-KD cells (Figures 4B and 4C). These observations indicate that TLR3 signaling enables efficient induction of pluripotency by using

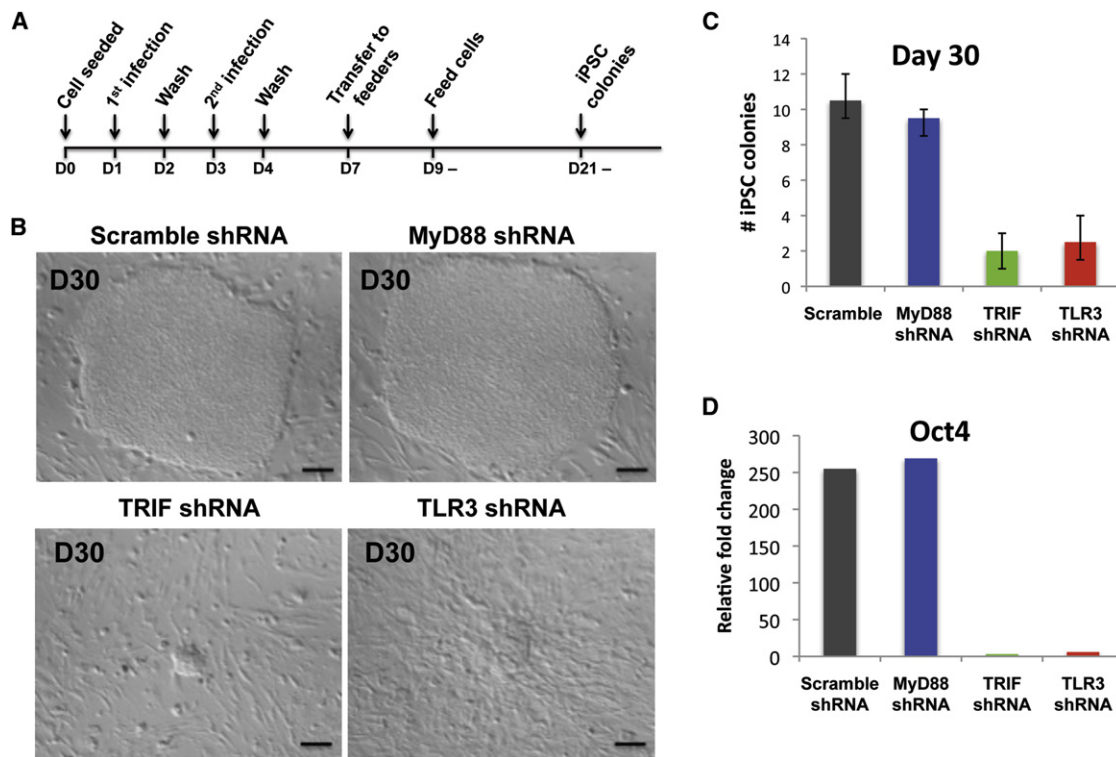


Figure 3. TLR3 or TRIF Knockdown Fibroblasts Exhibit Impaired Nuclear Reprogramming

(A) Protocol for iPSC generation using the reprogramming factors, delivered as retroviral vectors.

(B) Representative images of iPSCs on day 30 after initiation of retroviral nuclear reprogramming for scramble, MyD88, TRIF, and TLR3 shRNA knockdown fibroblasts. In fibroblasts where the TLR3 signaling pathway was knocked down (i.e., TRIF shRNA or TLR3 shRNA cell lines), the development of human iPSC colonies was markedly delayed. By contrast, in fibroblasts where the adaptor for all other TLRs was knocked down (MyD88 shRNA), no delay in hiPSC development was noted.

(C) Total number of hiPSC colonies on day 30 in scramble, MyD88, TRIF, and TLR3 shRNA knockdown fibroblast cell lines transduced by the reprogramming factors delivered by retroviral transfection. The yield of hiPSC colonies was reduced by knocking down the TLR3 signaling pathway. All data represented as mean \pm S.D., * $p < 0.05$; scramble compared to TRIF or TLR3 shRNA knockdown fibroblasts.

(D) Fold change in *Oct4* gene expression in scramble, MyD88, TRIF, and TLR3 shRNA knockdown fibroblasts at day 35.

mmRNA, as well as retroviral vectors, encoding the reprogramming factors.

TLR3 Agonist Accelerates CPP-Induced Target Gene Expression

If TLR3 activation plays a role in the efficiency of viral-based reprogramming, then the addition of a TLR3 agonist would be predicted to enhance CPP-induced reprogramming. Polyinosinic-polycytidylic acid (Poly I:C) is a synthetic analog of dsRNA that is recognized specifically by TLR3 and which induces the expression of genes involved in innate immunity (data not shown) (Alexopoulou et al., 2001). Accordingly, we assessed the effect of the CPPs alone or in the presence of poly I:C. The expression of target genes was unaffected by poly I:C alone. However, when poly I:C (300 ng/ml) was combined with CPP-SOX2, the expression of the downstream genes was accelerated, reproducing the time course of gene expression induced by pMX-Sox2 (Figures 5A and 5B). We repeated these studies with CPP-OCT4. When poly I:C was combined with CPP-OCT4, again the temporal expression of the downstream genes was accelerated, mimicking the effect observed with the viral

vector pMX-Oct4 (Figures 5C and 5D). Furthermore, as seen in Figures S2A–S2C, the effect of poly I:C to enhance gene expression of CPP-OCT4 was maintained at later time points. These studies supported the hypothesis that TLR3 signaling is required for efficient induction of the target genes of the reprogramming factors.

TLR3 Activation Enhances Efficiency of a Doxycycline-Inducible System for Generating iPSCs

To further test the hypothesis that TLR3 activation was required for efficient reprogramming, we isolated MEFs from murine embryos expressing a doxycycline (dox)-inducible polycistronic transgene construct encoding the four reprogramming factors (Wernig et al., 2008). To generate iPSCs, 10^5 MEFs/per well in 6-well plates were treated with dox. In some wells, poly I:C was also added for the initial 6 days of the reprogramming process. In other wells, cells were infected with pMX-GFP on the first day of dox treatment. Poly I:C or pMX-GFP each accelerated the dox-induced expression of *Oct4* and *Sox2* (Figure 6A). Poly I:C as well as pMX-GFP accelerated changes in the morphology of the MEFs with small, compact rounded cells

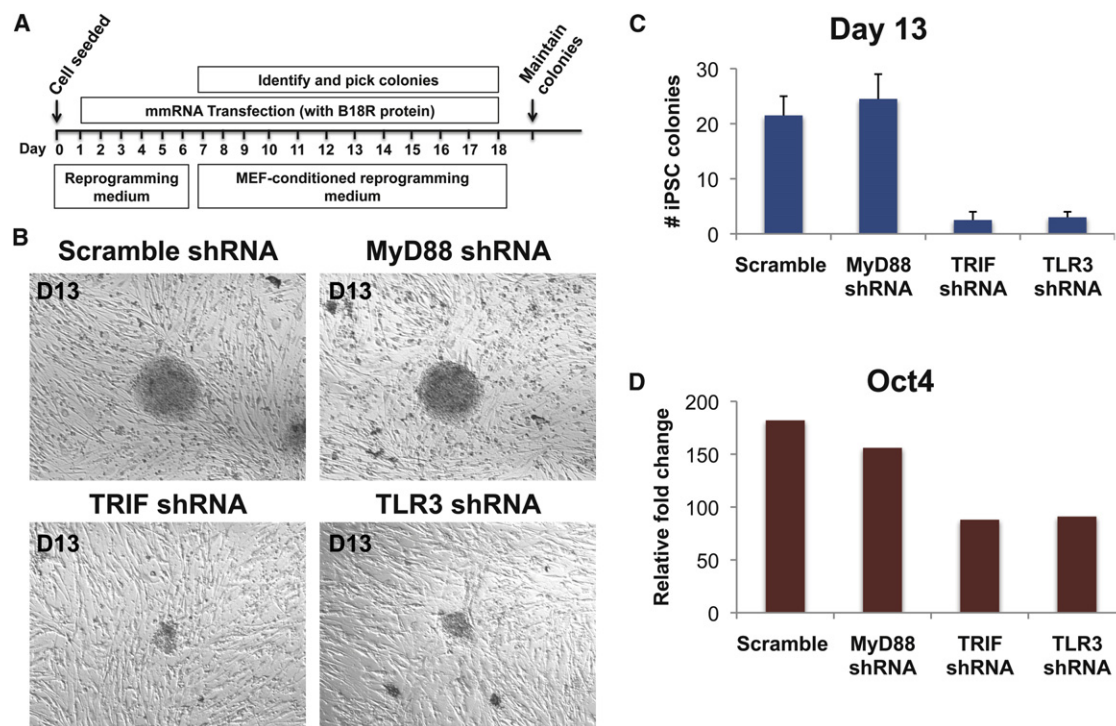


Figure 4. Nuclear Reprogramming Using mmRNA Is Inhibited by TLR3 or TRIF Knockdown

(A) Protocol for iPSC generation using the reprogramming factors, delivered as mmRNA.

(B) Representative images of human iPSCs on day 13 after initiation of mmRNA nuclear reprogramming in the scramble, MyD88, TRIF, and TLR3 shRNA knockdown fibroblasts. In fibroblasts where the TLR3 signaling pathway was knocked down (i.e., TRIF shRNA or TLR3 shRNA cell lines), the development of human iPSC colonies was markedly delayed. By contrast, in fibroblasts where the adaptor for other TLRs was knocked down (MyD88 shRNA), no delay in hiPSC development was noted.

(C) Total number of hiPSC colonies on day 13 in scramble, MyD88, TRIF, and TLR3 shRNA knockdown fibroblast cell lines transduced by the mmRNA reprogramming factors. The yield of hiPSC colonies was reduced by knocking down the TLR3 signaling pathway. All data represented as mean \pm S.D., * $p < 0.05$; scramble compared to TRIF or TLR3 shRNA knockdown fibroblasts.

(D) Fold change in Oct4 gene expression in individual colonies derived from scramble, MyD88, TRIF, and TLR3 shRNA knockdown fibroblasts at day 13 of their mmRNA reprogramming.

aggregating in the wells at 3 days (data not shown). Similarly, infection with pMX-GFP accelerated colony formation, as a number of small colonies were observed by day 7 in the viral particle infected group (data not shown). By day 14, typical mES-like colonies appeared, many of which expressed SSEA-1. At this time point, the number of typical SSEA-1⁺ colonies were increased by 7- to 8-fold in wells exposed to viral particles or poly I:C (Figure 6B). Colony number increased further by days 21–28 (Figures 6B and 6C). These studies strengthened the hypothesis that TLR3 activation enhances nuclear reprogramming to pluripotency.

TLR3 Activation Enhances CPP-Induced Generation of Human iPSCs

It is known that persistent expression (about 2 weeks) of the reprogramming factors using viral vectors is required to generate mouse iPSCs (Brambrink et al., 2008; Wernig et al., 2008). We hypothesized that activation of the TLR3 pathway might facilitate epigenetic alterations required for full transcriptional effect of the CPPs. Accordingly, we exposed human fibroblasts to the four CPP-transcription factors (OCT4-R11,

SOX2-R11, KLF4-R11, and CMYC-R11) (at a dose of 200 nM for days 1–6, and a dose of 100 nM for days 7–21), in the presence or absence of poly I:C (300 ng/ml) for days 1–6 (Figure 7A). The cultures were transferred to feeder cells (inactivated MEFs) at day 26. In the presence of poly I:C, Oct4 expression was accelerated (Figure 7B). Furthermore, poly I:C accelerated iPSC generation (Figures 7C and 7D). Small colonies were observed by day 21 and ES-like colonies appeared by day 30, many of which expressed TRA-1-81 as indicated by live cell staining. By contrast, in human fibroblasts not exposed to poly I:C, colonies were not observed until day 30. By day 40, colony number was increased by more than 4-fold in cells exposed to poly I:C (Figure 7C). Human iPSCs induced by CPP alone (piPSC) or in combination with poly I:C (ppiPSCs) manifested characteristic ESC morphology, immunoreactivity for SSEA3 and OCT4, and transcriptional expression (i.e., *Pou5f1*, *Sox2*, *Utf1*, *Zfp42*, and *Nanog*), and differentiated into all three embryonic germ layers in vitro and in teratomas (Figure S3). Thus, CPP-induced reprogramming to pluripotency of human fibroblasts was enhanced and accelerated by stimulation of TLR signaling.

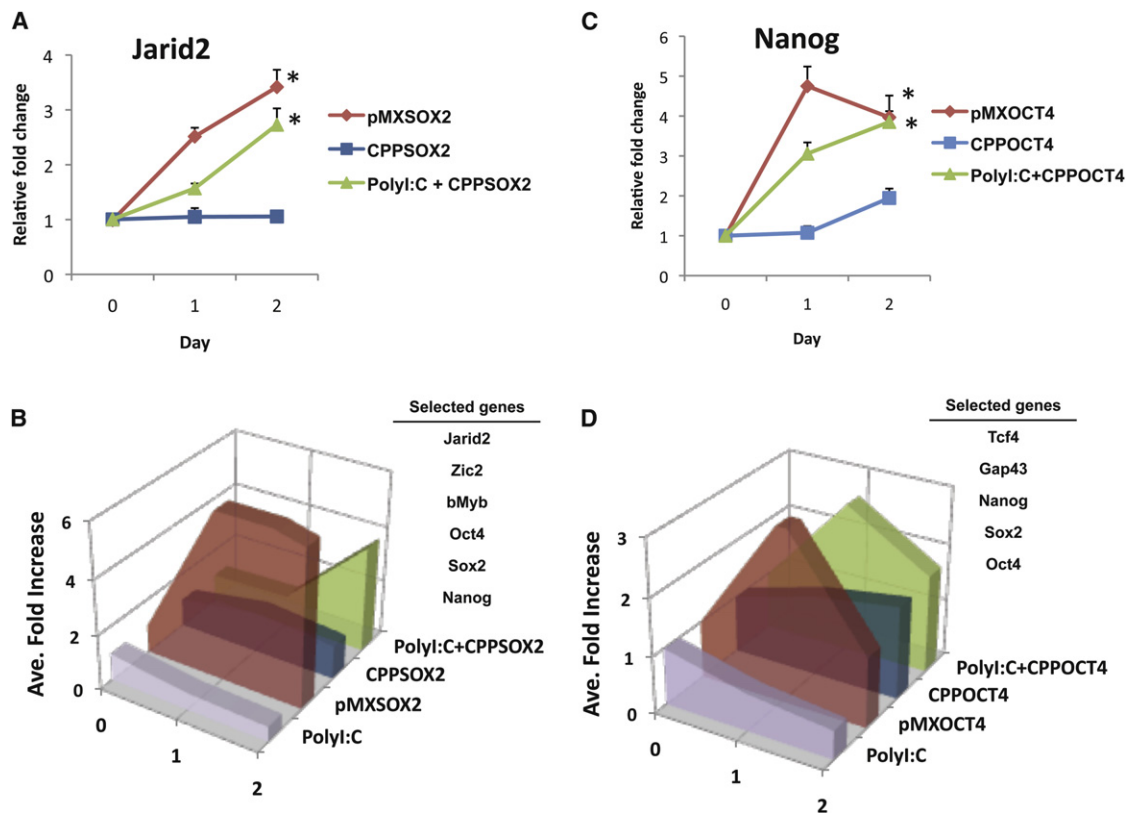


Figure 5. Poly I:C Accelerates CPP-Induced Target Gene Expression

(A) Relative fold change in gene expression levels of *Jarid2* following pMX-Sox2 (red line), CPP-Sox2 (blue line), or poly I:C + CPP-Sox2 (green line) treatments. (B) Summary figure of these selected genes (i.e., *Jarid2*, *Zic2*, *bMyb*, *Oct4*, *Sox2*, and *Nanog*) exhibiting the temporal pattern of average gene expression following each treatment. Poly I:C markedly enhances the expression of downstream genes by CPP-Sox2.

(C) Relative fold change in gene expression levels of *Nanog* following pMX-Oct4, CPP-Oct4, or poly I:C + CPP-Oct4 treatments.

(D) Summary figure of these selected genes (i.e., *Tcf4*, *Gap43*, *Nanog*, *Sox2*, and *Oct4*) exhibiting the temporal pattern of gene expression following each treatment. Poly I:C markedly enhances the expression of downstream genes by CPP-Oct4. All data represented as mean \pm S.D., $n = 3$, * $p < 0.005$ (see also Figures S4, S5, S6, and S7).

TLR3 Activation Enables Epigenetic Changes that Favor Reprogramming

We hypothesized that TLR3 activation might enhance early transcriptional activation by inducing an open chromatin state, permitting the reprogramming factors to induce an ESC-specific gene expression pattern (Niwa, 2007a, 2007b). Accordingly, we performed chromatin immunoprecipitation followed by PCR analysis (ChIP-PCR) to detect trimethylation of histone H3 at lysine 4 (H3K4me3). This epigenetic modification marks transcriptionally active genes. Human fibroblasts were treated with pMX-Sox2 or with CPP-Sox2 in the presence of poly I:C or pMX-GFP. By day 2 of treatment, pMX-Sox2 induced H3K4 trimethylation at the Oct4 promoter (Figure S4A). By contrast, CPP-Sox2 had no effect at day 2, except in the presence of viral vector or poly I:C (Figure S4A). (At later time points, CPP-Sox2 alone could induce H3K4 trimethylation, a time lag that reflected its delayed effects on target gene expression [data not shown]). Similarly, poly I:C, or the retroviral vector encoding GFP, accelerated H3K4 trimethylation at the Sox2 promoter in CPP-treated fibroblasts (Figure S5A).

In a similar fashion, we assessed histone H3 at lysine 9 (H3K9me3) in the Oct4 and Sox2 promoters. This epigenetic modification marks transcriptionally silenced genes. By day 2 of treatment, pMXSox2 but not CPPSOX2 alone, fully reversed H3K9 trimethylation at the Oct4 promoter (Figure S4B). Poly I:C or pMX-GFP enhanced the reversal of H3K9 trimethylation induced by CPP-Sox2 on day 2 (Figure S4B). (At later time points, CPP-Sox2 alone could fully reverse H3K9 trimethylation at the Oct4 promoter after a time lag that reflected its delayed effects on target gene expression [data not shown]). Similarly, poly I:C, or the retroviral vector encoding GFP, accelerated the loss of H3K9 trimethylation at the Sox2 promoter in CPP-treated fibroblasts (Figure S5B). These studies provided an epigenetic mechanism to explain the effect of TLR3 activation to enhance nuclear reprogramming.

TLR3 Activation Regulates Epigenetic Machinery

Histone acetylation status influences the accessibility of DNA to the transcriptional machinery for gene expression. Histone deacetylation is generally associated with a closed chromatin state,

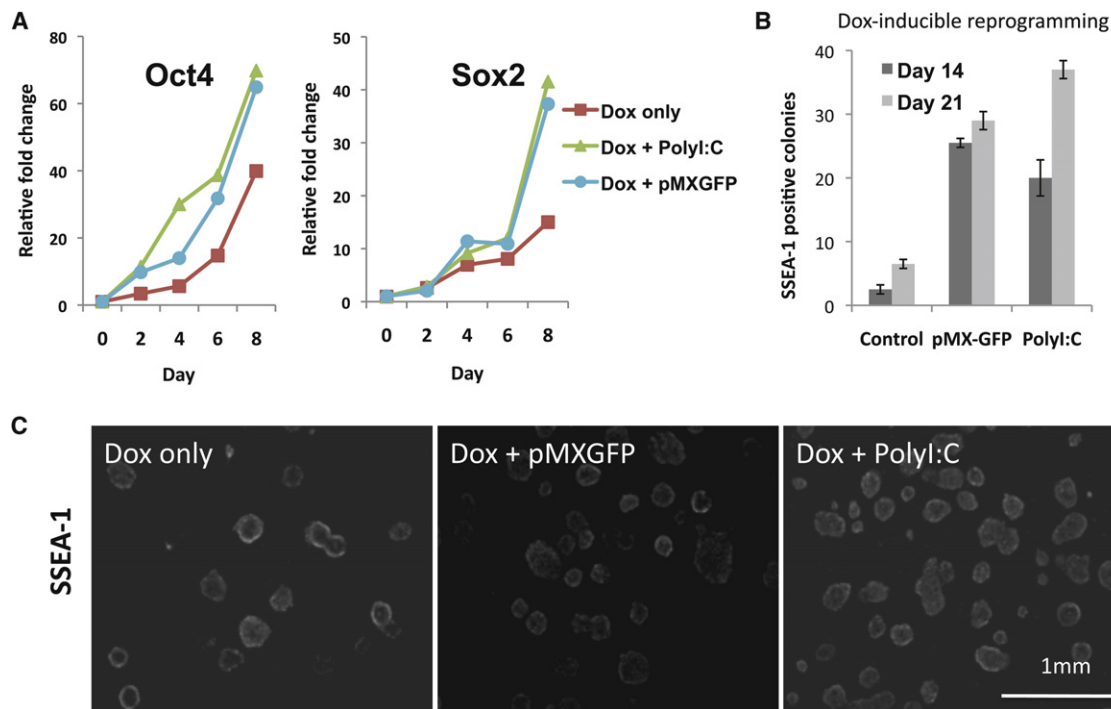


Figure 6. TLR3 Activation Enhances Reprogramming in a Dox-Inducible System

MEFs containing the dox-inducible polycistronic transgene construct encoding the four reprogramming factors were stimulated by dox, in the absence or presence of TLR3 activation with poly I:C or the irrelevant retroviral vector pMX-GFP.

(A) Gene expression of *Oct4* and *Sox2* was accelerated by coadministration of poly I:C, or a retroviral construct encoding GFP.

(B) Histogram showing SSEA-1+ colonies at 2 and 3 weeks in primary plates. Coadministration of poly I:C, or a retroviral construct encoding GFP, markedly increased the yield of dox-induced reprogramming.

(C) SSEA-1 live staining showing iPSC colonies derived from dox-inducible MEFs 4 weeks after exposure to dox. In some wells, MEFs were also exposed to a retroviral construct encoding GFP, or to poly I:C (see also Figures S6 and S7).

and inhibitors of histone deacetylase (HDAC) such as valproic acid are employed to enhance nuclear reprogramming. Therefore it is notable that poly I:C downregulated the expression of a suite of HDAC genes in CPP-treated human fibroblasts (Table S1). The downregulation of HDAC1 expression by poly I:C or by pMX-GFP was confirmed by western analysis (Figures S4C and S4D). Similar downregulation of the HDAC family by poly I:C was noted in the dox-inducible MEFs described above and in Figure 5. To determine whether the effect of poly I:C on epigenetic modifiers was dependent upon TLR3 signaling, we repeated these studies in the TLR knockdown lines. As anticipated, poly I:C reduced HDAC1 expression in the scrambled or Myd88 KD lines, but failed to downregulate HDAC1 expression in the TRIF- and TLR3-KD lines (Figure S5C). These studies indicated that activation of TLR3 signaling causes changes in epigenetic modifiers that favor reprogramming.

Poly I:C affected the expression of other epigenetic modifiers (Table S1). In addition, the changes in methylation status of the *Oct4* and *Sox2* promoters that were accelerated by TLR3 activation were also associated with an accelerated redistribution of heterochromatin protein 1 (HP1; data not shown). HP1 bound to methylated H3K9 recruits the methylase Suv39h, leading to further methylation of H3K9, so as to consolidate a repressed state (Bannister et al., 2001; Lachner et al., 2001). The redistribu-

tion of HP1 induced by poly I:C is consistent with genome-wide epigenetic alterations promoted by TLR3 activation, and directed by the reprogramming factors.

Role of NF- κ B in TLR-Mediated Regulation of Epigenetic Machinery

Histone acetylation favors an open chromatin state, maintained by proteins containing histone acetyltransferase (HAT) domains, such as p300 and CBP. NF- κ B is a transcriptional effector of TLR3 activation (Hayden and Ghosh, 2004; Hayden et al., 2006) and interacts with CBP/p300 to positively regulate gene expression (Farlik et al., 2010; Li and Verma, 2002). We used a luciferase reporter assay system to document that poly I:C, but not the CPPs alone, induced NF- κ B activation (Figure S6A). This effect was mediated by TLR3, as shRNA knockdown of TLR3 or its adaptor protein TRIF reduced the effect of poly I:C on NF- κ B activation (Figure S6A). Similarly, the retroviral constructs, as well as poly I:C, induced a sustained increase in the expression of NF- κ B and *Tlr3*, whereas CPP-SOX2 did not (Figure S6A). These studies suggest that the effect of TLR3 activation to induce changes in gene expression of the epigenetic machinery might be mediated in part by NF- κ B.

To further elucidate the elements of TLR3 signaling involved in reprogramming, we inhibited the action of downstream effectors

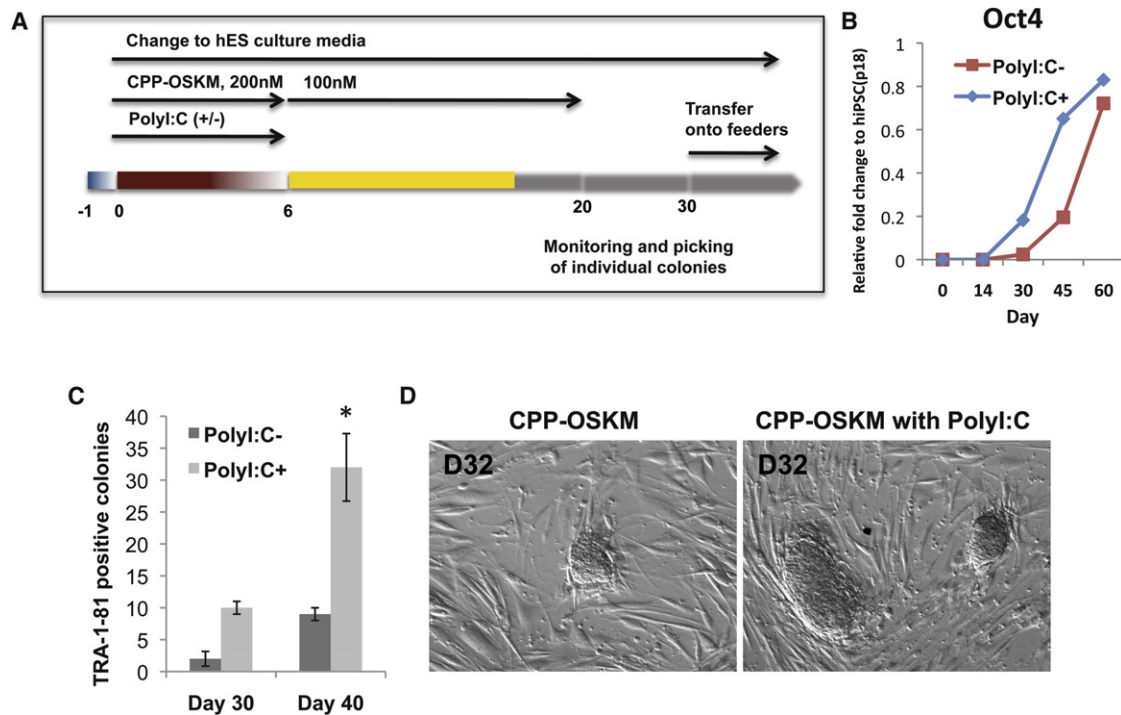


Figure 7. TLR3 Activation Stimulates CPP-Induced Reprogramming of Human Fibroblasts

(A) Protocol for human iPSC generation using four CPP-TFs (OCT4-R11, SOX2-R11, KLF4-R11, and cMYC-R11).

(B) Gene expression of *Oct4* was increased by coadministration of poly I:C, by day 30–45.

(C) TRA-1-81 positive colonies counted at day 30 and day 40 in the presence and absence of poly I:C. Coadministration of poly I:C markedly increased the yield of CPP-induced reprogramming. All data represented as mean \pm S.D., $n = 3$, * $p < 0.01$.

(D) ES-like colony formation at day 32 of CPP-induced transactivation (10x) (see also Figures S3, S4, S5, S6, and S7 and Table S1).

IKK ϵ , NF- κ B, and IRF3. As before, we found that poly I:C enhanced reprogramming of dox-inducible MEFs. This effect of poly I:C to induce NF- κ B translocation to the nucleus (Figure S6B), and to increase colony formation (Figure S6D), was markedly reduced by the addition of p65 decoy. In additional experiments, shRNA knockdown of IRF3 or IKK (Figure S6C) also impaired iPSC generation (Figure S6D). These studies indicated that TLR3-induced activation of both NF- κ B and IRF3 were required for efficient reprogramming.

To further document a role for these transcriptional factors in TLR3-facilitated chromatin modification, we performed ChIP assay for H3K4me3 and H3K27me3. We observed that the inhibition of NF- κ B, IRF3 or IKK ϵ fully eliminated H3K4 trimethylation at the *Oct4* promoter after dox stimulation in the presence of poly I:C (Figure S7A). Furthermore, H3K27 trimethylation of the *Oct4* promoter was increased upon inhibition of the innate immune pathway (Figure S7B).

In the reprogramming dox-inducible MEFs, the addition of poly I:C induced changes in the expression in a set of enzymes involved in chromatin modification (Figure S7C). We observed that poly I:C upregulated histone acetyltransferase (i.e., *Hat1* and *Eso2*) and histone methyltransferase (*Smyd*) as well as downregulated the histone deacetylase family (i.e., *Hdac* 5, 8, 9, and 10), H3K4 histone methyltransferase (*Ash1l*) and H3K79 histone methyltransferase (*Dot1l*). Inhibition of the latter methyl-

transferase is critical in somatic cell reprogramming (Onder et al., 2012). Notably, the effects of poly I:C on these epigenetic modifiers was reversed by decoy oligonucleotide inhibition of NF- κ B (Figure S7D).

DISCUSSION

This report posits a direct link between nuclear reprogramming efficiency and inflammatory pathways in the induction of pluripotency. In seeking to induce pluripotency while employing CPPs, we serendipitously discovered a role for innate immunity signaling in effective nuclear reprogramming. Our salient observations are: (1) A consistent difference in the temporal characteristics of gene expression is observed between cells exposed to the reprogramming factors in the form of retroviral vectors versus CPPs. (2) This difference is reversed by combining CPPs with TLR3 agonists. (3) TLR3 knockdown inhibits the activation of downstream pluripotency genes when using retroviral vectors or mmRNA to overexpress the reprogramming factors and reduces the efficiency and yield of human iPSC generation. (4) TLR3 activation enhances the efficiency and yield of miPSC generation in a dox-inducible system; and enhances the efficiency and yield of human iPSC generation when using the reprogramming factors in the form of CPPs, and (5) TLR3 activation enables epigenetic alterations, including changes in methylation

status of the Oct4 and Sox2 promoters as well as changes in the expression of epigenetic effectors, that promote an open chromatin configuration. The knowledge that the activation of innate immune response affects nuclear reprogramming permitted us to enhance the efficiency and yield of human iPSCs by using reprogramming factors in the form of CPPs.

An Unappreciated Role for TLR3 Activity in Reprogramming

TLR3 recognizes double-stranded RNA (dsRNA) generated by retroviruses (Agger et al., 2007; De Santa et al., 2007). The importance of TLR signaling for effective nuclear reprogramming has not been appreciated. We show that the efficiency and yield of human iPSC generation, using retroviral vectors, is reduced by knockdown of the pathway with peptide inhibitors or shRNA knockdown of TLR3 or its adaptor protein TRIF. This effect of TLR3 knockdown is also observed when using mmRNA to express the reprogramming factors.

We confirmed the importance of TLR signaling in a virus-free system by using murine embryonic fibroblasts that were genetically engineered to express a dox-inducible cassette encoding the reprogramming factors. In this system, the coadministration of the TLR3 agonist poly I:C increased the efficiency and yield of murine iPSCs (Figure 6). Notably, the same effect was observed with coadministration of the retrovirus encoding GFP, which retrovirus would be expected to activate TLR3 without otherwise affecting the nuclear reprogramming process. Our work indicates that the retroviral vectors used for inducing pluripotency are more than vehicles for delivering the reprogramming factors and actively contribute to the reprogramming process.

It is worth noting that there seems to be an optimal level of TLR3 activation. Because retroviral vectors activate the TLR3 pathway, further activation does not seem to be necessary during reprogramming with retroviral vectors and may even be counterproductive. Higher levels of activation are associated with less colony formation, probably due to cell death (data not shown).

TLR3 and Epigenetic Modification

The effect of TLR3 activation to enhance the yield and efficiency of human iPSC generation is due in part to its regulation of the expression and/or distribution of epigenetic modifiers (Table S1; Figures 7, S4C, S4D, and S5C). Associated with the changes in expression of epigenetic modifiers, we observed histone modifications consistent with an open chromatin configuration on the promoter regions of Oct4 and Sox2 (Figures S4 and S5). Notably, however, the increase in H3K4 trimethylation and the decrease in H3K9 trimethylation of these promoters were not observed with TLR3 activation alone. Only in the presence of the CPPs did poly I:C induce changes in these methylation marks. This observation supports the concept that, although TLR3 activation causes widespread changes in the expression of epigenetic modifiers that might promote the open chromatin configuration of pluripotency genes, the reprogramming proteins are likely necessary to direct the epigenetic modifiers to the appropriate promoter sequences. This notion is also supported by the confocal images of HP1 α distribution (data not shown).

Heterochromatin protein1 (HP1) is associated with the closed conformation of chromatin. Although we did not see changes in the expression levels of HP1 expression (Figures S4C and S4D), we observed marked changes in its distribution when CPP-SOX2 was coadministered with poly I:C or with the retroviral vector encoding GFP. However, in the absence of the CPP, there was no observable redistribution of HP1 induced by poly I:C or the retroviral vector alone.

Any of five classes of pathogen recognition receptors (PRRs) have been shown to signal in ways that are comparable to TLR3 (Roy and Mocarski, 2007) and might be expected to accelerate nuclear reprogramming. The fact that the current work implicated TRIF (Choe et al., 2005) signaling aligns with the fact that the retrovirus RNA, or the mmRNA, triggers TLR3 signaling to engage NF- κ B, IRF3, and IFN β . Other TLR or PRRs drive a similar inflammatory response converging on NF- κ B, IRF3, and IFN β (Akira and Takeda, 2004).

Type 1 interferons (e.g., IFN β) are elaborated during exposure to mmRNA or viral vectors, and we have not ruled out their role in reprogramming efficiency. In addition, silencing of endogenous retrotransposons is characteristic of the fully reprogrammed pluripotent state (Brambrink et al., 2008; Stadtfeld et al., 2008; Wernig et al., 2008). Notably, IRF3 induces expression of the tripartite motif (TRIM) protein family, which is implicated in silencing of endogenous retroelements in ES cells (Wolf and Goff, 2007). We observed that poly I:C induces the expression of the TRIM family (Table S1), and this effect of TLR3 activation may play a role in the reprogramming process by silencing endogenous retrotransposons.

In any event, our data suggest that activation of inflammatory pathways enhance nuclear reprogramming. Innate immunity signaling appears to favor an open chromatin state, which increases cell plasticity in response to a pathogen. We speculate that this cell state may enhance induction of pluripotency, transdifferentiation, or even malignant transformation and is characterized by global changes in epigenetic modifiers in a process we term “transflammation.”

CONCLUSION

Our observations highlight a previously unrecognized role for innate immunity activation in nuclear reprogramming. The viral vectors or mmRNA constructs used to induce pluripotency are more than mere vehicles for the reprogramming factors. Innate immune activation causes striking changes in epigenetic modifiers that favor an open chromatin configuration. These changes enable a fluidity of cell phenotype that contributes to successful nuclear reprogramming.

EXPERIMENTAL PROCEDURES

Cells

BJ human fibroblast cells (Stemgent) were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin (5% CO₂, 37°C). For isolation of secondary dox-inducible MEFs, chimeric embryos were obtained from transgenic R26^{rtTA};Col1a1^{2lox-4F2A} mice expressing the loxP-flanked, dox-inducible polycistronic 4F2A cassette (Oct4, Sox2, Klf4, c-Myc; Jackson Laboratory). Secondary MEFs were isolated as previously described (Wernig et al., 2008), and expanded for two passages before freezing.

Viral Preparation and Infection

HEK293FT cells were plated at 6×10^6 cells per T225 flask and incubated overnight. Cells were transfected with 10 μ g of VSV-G (envelope protein), 15 μ g of pUMVC (packaging plasmid), and 10 μ g of gene of interest (Sox2 or Oct4) with Lipofectamine. 48 hr after transfection, the supernatant of transfectant was collected and filtered (0.45 μ m filter). Following spinning at 17,100 rpm for 2.3 hr, the viral pellet was resuspended to make 100 \times stock solutions. Human fibroblasts (5×10^4 cells/well) were seeded a day before transduction. The medium was replaced with virus-containing supernatant supplemented with 8 μ g/ml polybrene, and incubated for 24 hr.

Treatments

At 60%–70% confluency, BJ fibroblast cells were serum-starved (1% serum) to induce G1 cell cycle arrest and then subjected to either a single infection with retroviral constructs or daily treatments with 200 nM CPPs (CPP-SOX2 or CPP-OCT4). Poly I:C (300 ng/ml) was added to the cells simultaneously with the CPPs. For experiments involving peptide inhibitors, cells were pre-treated for 6 hr at 40 μ M with either MyD88 inhibitory peptide (Pepinh-MyD) or TRIF inhibitory peptide (Pepinh-TRIF) followed by CPP treatments.

Gene Expression and Microarray Analyses

RNA was isolated (RNeasy), first-strand cDNA primed with oligo(dT) primers, and qPCR performed (primer sets from Applied Biosystems). RNA probes were prepared and hybridized to Illumina HumanHT-12 v4 Expression Bead-Chip microarrays.

Short Hairpin RNA Design

Short hairpin RNA was obtained from Invivogen. Target sequences: MyD88 shRNA, AACTGGAACAGACAACTATC; TRIF shRNA, AAGACCAGACGCCA CTCCAAC; and TLR3 shRNA, GCTTGGCTTCCAACTAGAA.

Chromatin Immunoprecipitation and Chromatin

Immunoprecipitation-qPCR

Quantitative chromatin immunoprecipitation (qChIP) was performed (Lim et al., 2009; Peng et al., 2009). For qChIP and qRT-PCR, error estimates are standard deviations. Recovery of genomic DNA as the percentage input was calculated as the ratio of copy numbers in the immunoprecipitate to the input control. Primers of Oct4 and Sox2 promoters were purchased from Cell Signaling.

Generation of iPSCs

Retroviral iPSCs

Human fibroblasts previously treated with MyD88, TRIF, TLR3, or Scramble shRNA were transduced with pMX-Oct4, pMX-Sox2, pMX-Klf4, and pMX-cMyc retroviruses (Takahashi et al., 2007; Takahashi and Yamanaka, 2006) and were cultured in iPSC medium on mitomycin-treated MEFs. Colonies were counted over time and were harvested for RNA isolation qPCR analysis for pluripotency gene expression.

Protein iPSCs

Recombinant Oct4, Sox2, Klf4, and cMyc human proteins (CPPs) contained an 11-arginine membrane penetration domain at the C terminus (Stemgent). Human fibroblasts were treated with CPP-OCT4, CPP-SOX2, CPP-KLF4, and CPP-cMYC daily for 6 days (200 nM), then daily from days 7–20 (100 nM). Poly I:C (300 ng/ml) or vehicle was added to the cell simultaneously up to day 6. The cells were passed onto MEF feeders at day 30. After 20 days of CPP treatments, wells were scanned for colonies.

Dox-Induced iPSCs

A total of 4×10^4 /well secondary MEFs (passage #4) were plated and treated with dox (2 μ g/ml) \pm poly I:C (300 ng/ml). The generation of iPSC colonies was scored at days 14 and 21.

Induction of Pluripotency Using mmRNA

Knockdown human fibroblasts were transduced with mmRNA encoding OSKM and were cultured in iPSC medium on mitomycin-treated MEFs (Yakubov et al., 2010). Colonies were counted by day 13, and single colonies were harvested for RNA isolation and qPCR analysis for pluripotency gene expression.

NF- κ B Luciferase Assay

BJ fibroblasts (3×10^5 /well) were subjected to either pMX-GFP infection or CPP-SOX2 treatment with or without poly I:C (300 ng/ml). Cells were transfected with pNF- κ B-Luc and pFC-MEKK as a positive control plasmid by using Lipofectamine 2000, and after 24 hr cells were studied by using the Bright-Glo Luciferase Assay System and a luminometer.

Immunostaining of Live Cells

For the detection of SSEA-1 or TRA-1-81 in live cells, the primary Ab (anti-mouse SSEA-1, anti-human TRA-1-81; Stemgent) was diluted to a final concentration of 2.5 to 5 μ g/ml in cell culture medium and incubated with cells for 30 min (37°C, 5% CO₂). After gentle washing, the cells were examined under a fluorescent microscope.

Western Blotting

Proteins were extracted from BJ fibroblasts by solubilizing the cells in RIPA buffer containing 1 \times protease inhibitor cocktail. Twenty-five micrograms of total protein was loaded and resolved on SDS-polyacrylamide gels, transferred to polyvinylidene fluoride membranes and probed with Abs against HP1 α , HDAC1 (Cell Signaling), and β -ACTIN (Sigma, A5441). Immunoblots were developed with enhanced chemiluminescence reagents (Amersham).

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2012.09.034>.

ACKNOWLEDGMENTS

We thank Drs. Joanna Wysocka, Karla Kirkegaard, and Vittorio Sebastiano for their guidance; Mr. Liqun Zhu for technical assistance; and Dr. Yaso Natkunam for histological studies. This work was supported by grants to J.P.C. from National Institutes of Health (U01HL100397 and RC2HL103400). J.L. was supported by grant from the Tobacco Related Disease Research Program of the University of California. N.S. was supported by NRSA grant (HL098049-01A1). J.P.C., N.S., and J.L. are inventors of the intellectual property, assigned to Stanford University, which was generated by this research.

Received: December 26, 2011

Revised: July 5, 2012

Accepted: September 18, 2012

Published: October 25, 2012

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